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Induction of IGFBP-1 expression by amino acid deprivation of HepG2 human hepatoma cells involves both a transcriptional activation and an mRNA stabilization due to its 3′UTR

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Abstract A dramatic overexpression of IGFBP-1 is responsible for growth inhibition, in response to a low-protein diet feeding. It has been demonstrated that a fall in the amino acid concentration was directly responsible for IGFBP-1 induction. In this report, we sought to determine the mechanism by which amino acid limitation upregulates IGFBP-1 expression. Our results show that both transcriptional activation and mRNA stabilization are involved. We also demonstrate that (i) the mGCN2/ATF4 pathway is not involved in this regulation and (ii) the 3′UTR of IGFBP-1 mRNA is responsible for its destabilization and regulates its stability in response to amino acid starvation.

Keywords: IGFBP-1; Amino acid; Transcription; mRNA stability; Gene expression

1. Introduction

Prolonged feeding on a low protein diet causes a fall of the plasma level of most essential amino acids. For example leucine and methionine concentrations can be reduced from about 100–150 and 18–30 μM to 20 and 5 μM, respectively, in plasma of children affected by kwashiorkor [1,2]. Because certain amino acids are not synthesized by the organism and because there is no amino acid storage, individuals have to adapt to this amino acid deficiency. One of the main consequences of feeding a low protein diet is the dramatic inhibition of growth in young individuals [3]. Straus et al. [3] demonstrated that an overexpression of IGFBP-1 was responsible for growth inhibition in response to a prolonged feeding on a low protein diet. IGFBP1 inhibits the mitogenic and metabolic effects of the growth factors IGF1 and IGF2.

IGFBP-1 expression is mainly regulated by GH, insulin or glucose. However, the high IGFBP-1 levels found in response to a protein deficient diet cannot be explained by these three factors. It has been demonstrated that a fall in the amino acid concentration was directly responsible for IGFBP-1 induction [3,4]. Starvation in any essential amino acid or in arginine or cysteine leads to a strong induction of IGFBP-1 mRNA and protein expression in a dose-dependent manner. Moreover, an amino acid limitation, as occurring in blood of protein-restricted animals, induces IGFBP-1 gene expression.

The current understanding of the molecular events involved in the amino acid regulation of gene expression is still limited. Amino acid availability is known to alter a number of cellular functions including transcription and translation. At the molecular level, only the transcriptional regulation of CHOP [5] and asparagine synthetase (AS) [6] have been investigated. Transcription of CHOP and AS increases in response to amino acid starvation. *cis*-Elements responsible for this regulation have been located in the promoter of these genes and termed amino acid responsive element (AARE) or nutrient sensing response element [7,8].

The pathway involved in the amino acid regulation of CHOP and AS expression involves the kinase mGCN2, which is activated by free tRNA accumulation during amino acid starvation. Once activated, mGCN2 phosphorylates the translation initiation factor eIF2α, impairing the synthesis of the 43S preinitiation complex and thus strongly inhibiting translation initiation. Under these circumstances, the transcription factor ATF4 is translationaly upregulated due to the presence of uORFs in its 5′UTR [9,10]. Then, ATF4 binds the AARE and induces the expression of CHOP or AS [11,12]. Other transcription factors have also the ability to bind the AARE. However, their role in the amino acid control of transcription is not established [11,12].

In this report, we sought to determine the mechanism by which an amino acid limitation upregulates IGFBP-1 expression. Our results demonstrate that both transcriptional activation and mRNA stabilization are involved.

2. Materials and methods

2.1. Cell culture and treatment conditions

HepG2 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% fetal calf serum (FCS). When indicated, DMEM lacking leucine was used. In all experiments involving amino acid starvation, dialyzed calf serum was used.

Mouse primary cultures of isolated hepatocytes were prepared as previously described [13].

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2.2. DNA transfection

Cells were transfected by the calcium/phosphate coprecipitation method as described previously [14]. Cells were exposed to the precipitate for 16 h and incubated for 1 min with 20% glycerol diluted in phosphate-buffered saline (PBS). Then, cells were washed twice in PBS, and incubated with DMEM containing 10% FCS. Twenty-four hours after transfection, cells were leucine starved and harvested.

2.3. RNA preparation

Total RNA were prepared using a RNeasy mini kit (Qiagen). RNA integrity was electrophoretically verified by ethidium bromide staining.

2.4. Analysis of gene expression using real-time RT-PCR

RNA were treated with DNase I, Amp Grade (Gibco-BRL Life Technologies) prior to cDNA synthesis. RNA (0.5 μg) were reverse transcribed with 100 U of Superscript II plus RNase H$^-$ Reverse Transcriptase (Gibco-BRL Life Technologies) using 100 nM random hexamer primers (Pharmacia Biotech.), according to the manufacturer's instructions. PCR was carried out using a LightCycler™ System (Roche) as described precedently [14]. Each experiment was repeated three times to confirm the reproducibility of the results.

Primers: IGFBP-1 mRNA quantification: (forward, 5′-cgcctgcgtgcaggagtctg-3′; reverse, 5′-agagccttcgagccatcata-3′); IGFBP-1 pre-mRNA: (forward, 5′-cccagagagcacggagataa-3′; reverse, 5′-cacagccgactcaagctaca-3′); *AS*: (forward, 5′-atcactgtcgggatgtaccc-3′; reverse, 5′-cttcaacagagtggcagcaa-3′); tagged IGFBP-1 (forward, 5′-gaacaaaagctgatatcggagga-3′; reverse, 5′-agagccttcgagccatcata-3′); human ATF4 (forward, 5′-ccccttcaccttcttacaacc-3′; reverse, 5′-gggctcatacagatgccacta-3′). To normalize for mRNA content, β-actin mRNA was also amplified. Primers for the human β-actin sequence: forward, 5′-tccctggagaagagctacga-3′; reverse, 5′-agcactgtgttggcgtacag-3′.

2.5. siRNA preparation and transfection

siRNA corresponding to the ATF4 mRNA sequence (5′-gccuaggucucuuagaugatt-3′ and 5′-ucaucuaagagaccuaggctt-3′) and a control siRNA (siCTL) corresponding to ATF4 siRNA (siATF4) mutated on three nucleotides (5′-gcguaguucgcuaaggugatt-3′ and 5′-ucaccuuagcgaacuacgctt-3′) were designed. This sequence is located 88 bp downstream the AUG codon. Transfection and experiments were performed as previously described [12].

2.6. Nuclear run-on transcription assays

In vitro transcription experiments in isolated HepG2 cell nuclei were carried out essentially as described by Bruhat et al. [14]. RNA were labeled with [^{32}P]UTP, then hybridized to filter-bound cDNAs of *IGFBP-1*, ribosomal S26 protein, and pBluescript DNA (Stratagene).

2.7. Plasmids constructions

A *Hin*dIII/*Sal*I fragment, containing the IGFBP-1 cDNA under the control of the CMV promoter, was cloned in place of the Luciferase gene in the pGl3 vector (Promega).

The tagged construct (IGFBP-1 tag) was generated by inserting the myc sequence (gaacaaaagctgatatcggaggaggacttg) in place of the gtgcaggagtctgacgcctccgctccccat sequence of IGFBP-1.

The IGFBP-1 tag Δ3′UTR construct was obtained by deletion of the PmlI cassette (containing the 3′UTR) from the IGFBP-1 tag construct.

The IGFBP-1 tagΔ 5′UTR construct was obtained as followed: A *Hin*dIII/*Sac*II fragment was generated by PCR amplification using the IGFBP-1 tag construct as a template, with the following primers: forward, 5′-tgtaagcttctcgcgcctggagatgtc-3′, reverse, 5′-ctctctgggctttcagggctc-3′. This fragment was then inserted into the *Hin*dIII/*Sac*II sites into the IGFBP-1 tag construct. All plasmids were sequenced before use.

2.8. Statistical analysis

All values are the means calculated from the results of at least three independent experiments. Data were analyzed by *t* test. Values presented are means ± S.E.M.

3. Results and discussion

3.1. Only part of the IGFBP-1 regulation in response to amino acid starvation occurs at a transcriptional level, and it does not involve the mGCN2/ATF4 pathway

A time course analysis of IGFBP-1 mRNA levels in HepG2 cells exposed to a medium lacking leucine indicates that mRNA content increases 4 h after starvation and that a maximum level plateaued after 16 h (Fig. 1A).

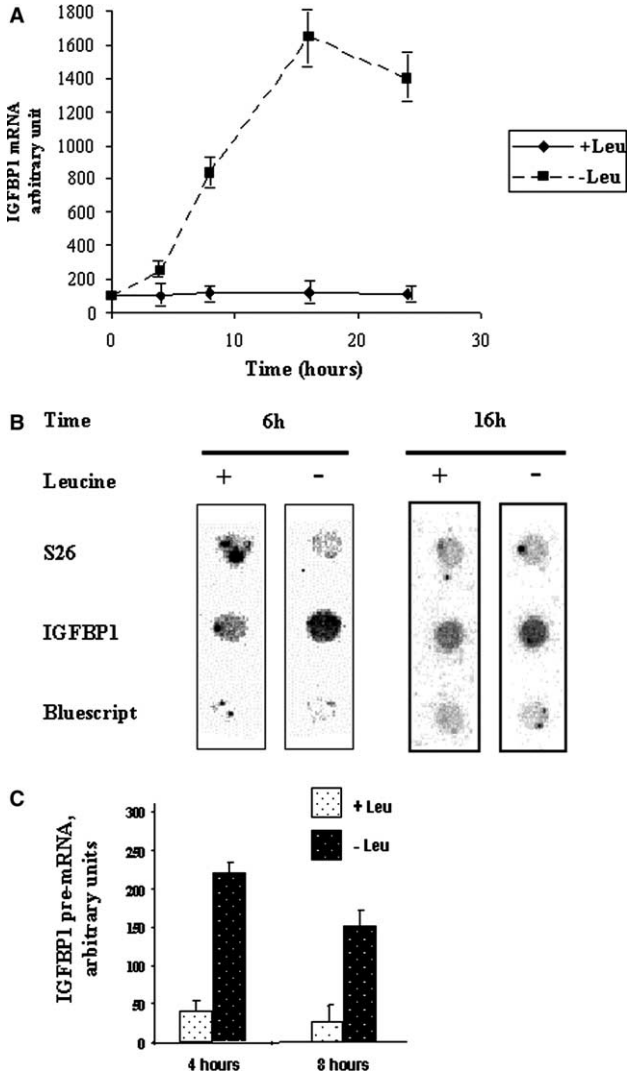

Fig. 1. Leucine starvation induces IGFBP-1 expression. (A) Induction of IGFBP-1 expression by leucine starvation. HepG2 cells were incubated in DMEM or in DMEM lacking leucine and harvested for RNA isolation after the indicated incubation time. Quantitative RT-PCR analysis were performed as described in Section 2. (B) Effect of leucine starvation on IGFBP-1 transcription. Nuclear run-on analysis of IGFBP-1 transcription: HepG2 cells were incubated for 6 and 16 h in DMEM control medium or in DMEM lacking leucine. Then nuclei were isolated and run-on experiments were performed as described in Section 2. ^{32}P-labeled RNA isolated from HepG2 cells were hybridized to filter-bound DNAs encoding ribosomal *S26*, IGFBP-1 and bluescript vector. (C) Induction of IGFBP-1 pre-mRNA by leucine starvation. The experiment was conducted as described in Fig. 1A. RT-PCR analysis were performed using the specific primers spanning an intron–exon junction in order to detect primary transcript containing intron3.

Transcriptional mechanisms appear to play an important role in the regulation of several amino acid regulated gene (CHOP, AS, etc.) [5,6]. From nuclear run-on experiments, we first investigated whether transcription was involved in the leucine regulation of IGFBP-1 expression. Fig. 1B shows that IGFBP-1 transcription is only transiently increased by a 6 h leucine starvation (about 2.5 times) whereas longer starvation (16 h) does not significantly increase the transcription rate (about 1.2) even though the mRNA continues to accumulate into the cells. It is also worth noticing that IGFBP-1 basal transcription rate is higher than the one of S26, indicating that IGFBP-1 basal transcription rate is sustained whatever the culture condition. Another way to investigate the change in the transcription rate of the IGFBP-1 gene consists in measuring the levels of unspliced IGFBP-1 pre-mRNA [11,15]. Quantitative RT-PCR analysis of IGFBP-1 pre-mRNA with specific primers spanning an intron–exon junction was used to amplify a transient intermediate. An increase in the IGFBP-1 pre mRNA transcript was observed after 4 and 8 h of leucine deprivation (Fig. 1C). It is noticeable that transcription is lower after 8 h leucine starvation than after 4 h. These data confirm that IGFBP-1 transcription is increased in response to leucine starvation.

Computer analysis of IGFBP-1 promoter suggests that it contains several putative AARE (not shown). In order to determine whether these sequences are involved in the amino acid regulation of IGFBP-1 gene expression, chimeric constructs containing up to 3.7 kb of the IGFBP-1 genomic region upstream the start site linked to the luciferase reporter gene were assayed for a response to amino acid deprivation. To investigate the possibility that an AARE could be located either in the coding sequence or in the first intron, a 4.9 kb genomic fragment containing 3.7 kb downstream the initiation site for transcription was cloned in front of the reporter gene and tested. All these constructs mediated not only a basal expression but also an insulin responsiveness to the Luciferase gene expression. However, none of them exhibited any significant increase in luciferase expression in response to amino acid starvation (data not shown). From these experiments we can conclude that, either the AARE is not located in the tested sequences, or a chomatine structure, that does not exist in transfected plasmids, is required for this regulation.

We can expect that several pathways could be involved in the amino acid control of IGFBP-1. Up to now, the mGCN2/ATF4 and mTOR pathways have been described as mechanisms by which amino acids regulate gene expression [16]. Both amino acid deprivation and rapamycin treatment inactivate the mTOR kinase. However, using transcriptional profiling, Peng et al. [16] showed rapamycin and amino acid starvation have similar effects on certain set of genes but have opposite effects on a large group of genes involved in the synthesis, transport and use of amino acids. Our results (not shown) and data from Cichy et al. [17] show that, in HepG2 cells, rapamycin treatment does not induce IGFBP-1 expression. To investigate whether the mGCN2/ATF4 pathway is also involved in the amino acid regulation of IGFBP-1 expression, we measured the effects of leucine starvation on IGFBP-1 expression in cell deficient for mGCN2 or ATF4. Small interfering double stranded RNA (siRNA) transfection was used to specifically inhibit the expression of ATF4. Fig. 2 shows that ATF4-siRNA transfection, while reducing the mRNA content of ATF4 (Fig. 2A), does not significantly affect the IGFBP-1 regulation by leucine starvation (Fig. 2B). As a control, we showed that ATF4 siRNA transfection prevents the induction of AS by amino acid deprivation (Fig. 2C).

To assess the role of mGCN2 in the regulation of IGFBP-1 by leucine availability, we used primary culture of hepatocytes isolated from mice deficient for mGCN2 and their corresponding wild-type cells. As shown in Fig. 3, IGFBP-1 is induced by leucine starvation in both wild type and mGCN2−/− cells whereas AS is not induced in the mGCN2−/− cells. Therefore, unlike *CHOP* or *AS* genes, the mGCN2/ATF4 pathway is not involved in the IGFBP-1 regulation by leucine availability.

3.2. *IGFBP-1 mRNA is stabilized in amino acid depleted cells by a sequence located in the 3′-UTR*

We have shown that the rate of IGFBP-1 transcription is only transiently increased by leucine starvation whereas the mRNA continues to accumulate into the cell. Therefore, it is likely that transcriptional regulation is not the unique mechanism involved in the amino acid control of IGFBP-1 expression. We can thus hypothesize that amino acid depletion increases IGFBP-1 expression by stabilizing its mRNA. To investigate the effects of leucine starvation on IGFBP-1 mRNA stability, we have not used the classical technique that consists of measuring mRNA decay after transcription inhibition by Actinomycin D. Indeed, the basal IGFBP-1 mRNA level is very low; as a consequence, it would have been necessary to previously induce its expression to measure accurately the decay. The treatment (amino acid starvation, hypoxia, serum depletion, etc.) able to induce IGFBP-1 expression also inhibits protein synthesis. It is likely that the

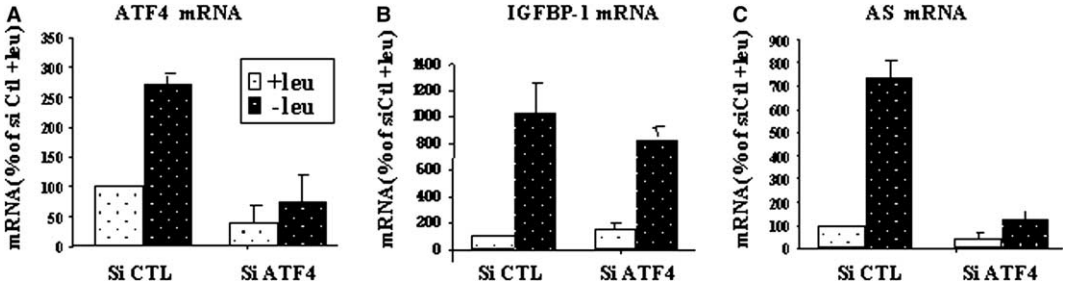

Fig. 2. Effect of ATF4 knockdown on the amino acid regulation of IGFBP-1 expression. HepG2 cells were transfected with ATF4 siRNA (siATF4) or control siRNA (siCTL). Two days after siRNA transfection, cells were incubated for 16 h in DMEM (+leu) or in DMEM lacking leucine (−leu) and then harvested. RNA were then extracted. The (A) ATF4, (B) IGFBP-1 and (C) AS mRNA levels were quantified by real-time RT-PCR.

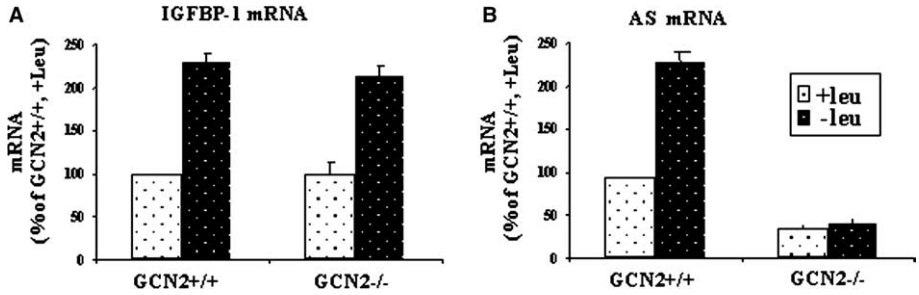

Fig. 3. The mGCN2 kinase is not involved in the amino acid regulation of IGFBP-1. Primary culture of isolated hepatocytes from GCN2 −/− and +/+ mice were used. After isolation, cells were plated in 6 cm dishes and cultured for 2 days. Then, cells were incubated for 16 h in DMEM (+leu) or in DMEM lacking leucine (−leu) and harvested for (A) IGFBP-1 and (B) AS mRNA determination.

mechanism involved in the regulation of the IGFBP-1 mRNA stability would have been turn-on during the induction period, making the experiment difficult to interpret. Therefore, we measured IGFBP-1 mRNA stability using a tagged IGFBP-1 cDNA driven by an heterologous promoter not regulated upon leucine starvation. HepG2 cells were transfected with the tested construct and then incubated for 8 h either in a control or starved medium. RNA were isolated and analyzed by RT-PCR for the expression of both the endogenous and the tagged IGFBP-1 transcripts. Fig. 4B shows that both the endogenous and the tagged IGFBP-1 mRNA expressions are strongly induced by leucine starvation. We can conclude that the IGFBP-1 transcript is stabilized by amino acid starvation. To identify the region of the mRNA responsible for the regulation of its stability, we deleted the 5′UTR or the 3′UTR. Deletion of the 3′UTR stabilizes the transcript and abolishes its amino acid regulation whereas the lack of the 5′UTR does not significantly affect the regulation of the tagged IGFBP-1.

In a previous study, Ooi et al. [18] showed that protein synthesis inhibition by a high dose of cycloheximide (CHX; 10.7 μM) impairs both transcription and transcript

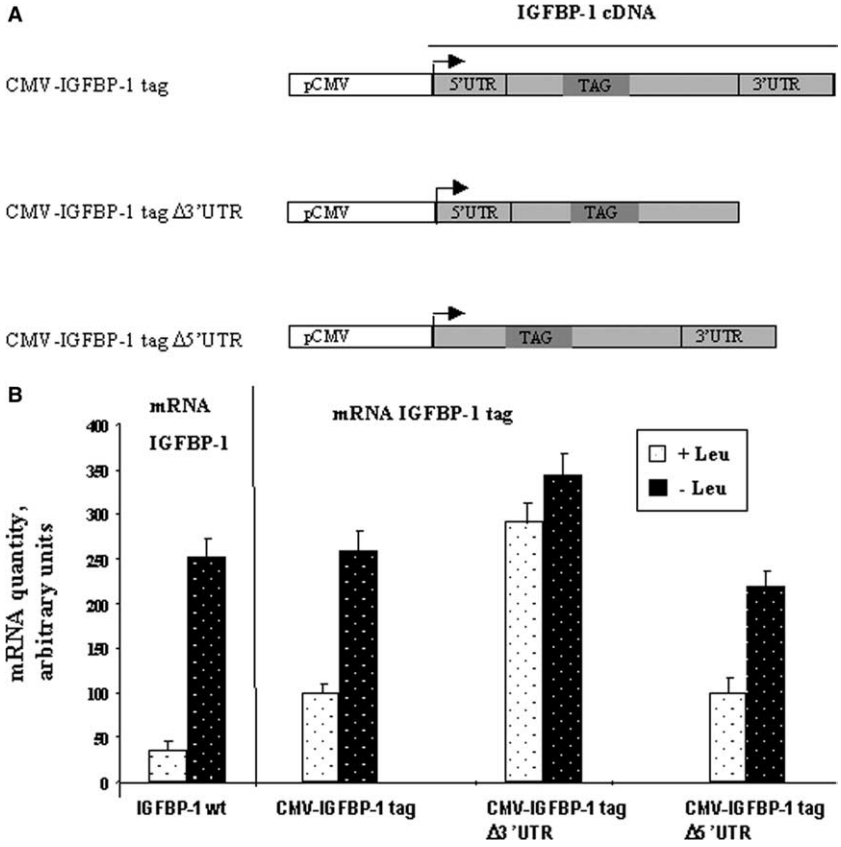

Fig. 4. Increased stability of the IGFBP-1 mRNA in amino acid depleted cells involves *cis*-acting mRNA sequences located within the 3′UTR. (A) Schematic representation of the cDNAs used to study mRNA stability. The arrow indicates the start site for transcription. The tag is represented by a shaded box. (B) HepG2 cells were transiently transfected with the constructs described in (A). Twenty four hours after transfection, cells were incubated for 8 h in DMEM medium (+leu) or in DMEM lacking leucine (−leu) and then harvested for RNA extraction. The endogenous and tagged IGFBP-1 mRNA levels were quantified by real-time RT-PCR using the appropriate primers.

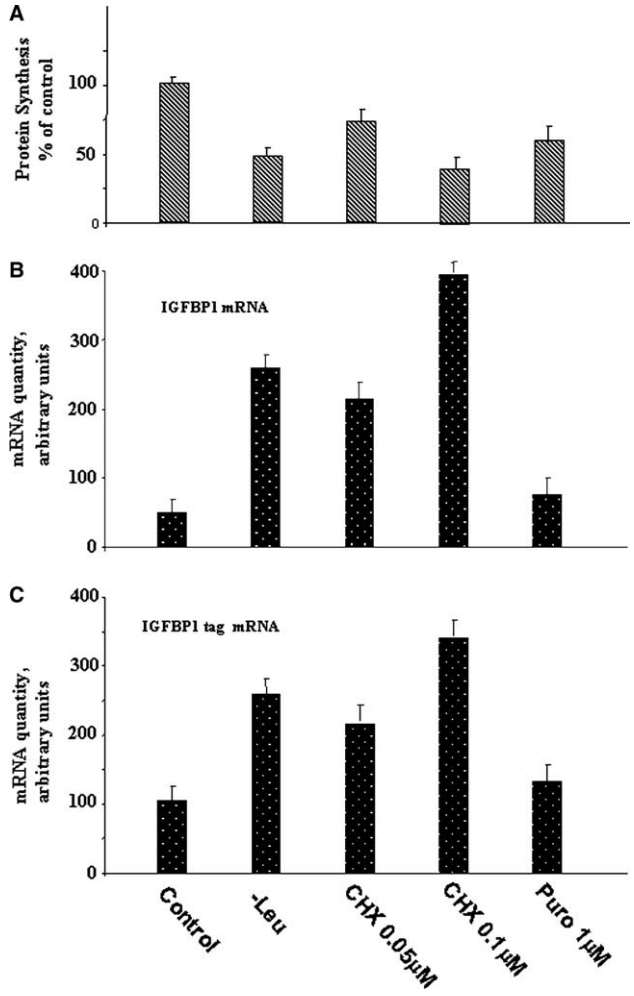

Fig. 5. Effect of protein synthesis inhibitors on the regulation of IGFBP-1 expression. HepG2 cells were transfected with the "IGFBP-1 tag" construct. Thirty six hours later cells were incubated for 8 h either in DMEM containing indicated concentrations of CHX, puro or in DMEM lacking leucine. Cells were then harvested for quantification of the endogenous and the tagged IGFBP-1 mRNA as previously described.

degradation of IGFBP-1. Therefore we investigated the potential link between mRNA degradation and protein synthesis during amino acid starvation, a process during which translation is inhibited. We used CHX and puromycin (puro) at low concentrations that do not inhibit completely transcription and that inhibit protein synthesis by about the same magnitude as leucine starvation does (Fig. 5A). CHX prevents the breakdown of the polyribosome mRNA complex whereas puro prematurely terminates the elongation of the polypeptide chain and thus reduces the density of ribosomes on the mRNA. Both CHX treatment and leucine starvation induce the expression of the endogenous (Fig. 5B) and tagged IGFBP-1 (Fig. 5C). Therefore, a low concentration of CHX stabilizes IGFBP-1 transcript. Unlike CHX, puro does not induce the expression of the endogenous or tagged IGFBP-1 transcript indicating that the transcript is not stabilized. Taken together these data suggest that association of the mRNA with ribosomes, rather than inhibition of translation per se, protects them from degradation.

4. Conclusions

In this work we show that leucine limitation leads to an induction of IGFBP-1 mRNA resulting from both a transient increase in the rate of transcription and an increase in the mRNA stability. Moreover, the GCN2/ATF4 pathway is not involved in the amino acid regulation of IGFBP-1 demonstrating that at least one other pathway is involved in the amino acid regulation of gene transcription.

Our results clearly establish that amino acid starvation increases the IGFBP-1 mRNA stability. We hypothesized that, in fed cells, the transcript is degraded very rapidly [18], whereas in starved cells, its degradation is somehow reduced and transcription transiently increased, resulting in an accumulation of the transcript. Although the mechanism involved in the regulation of mRNA turnover is not well understood, our work and other [18] show convincing evidences that destabilization of the IGFBP-1 transcript requires translation. We also demonstrate that the 3′UTR of IGFBP-1 transcript is responsible for the short half-life of the transcript and is involved in the amino acid regulation of its degradation. The IGFBP-1 mRNA is destabilized by five AU-rich element (ARE) within the 3′UTR [19]. We can hypothesize that the IGFBP-1 ARE are involved in the amino acid regulation of its mRNA stability. Further studies will be required to understand the molecular mechanisms involved in the amino acid control of IGFBP-1 mRNA stability. Particularly, the role of protein factors binding the ARE sequence should be investigated. It is noticeable that for most of the amino acid regulated genes (AS, CHOP and the cationic amino acid transporter, CAT1) that have been studied, it has been shown that the mRNA is more or less stabilized in response to amino acid depletion [14,20,21]. However, the molecular mechanisms involved in this process have not been identified.

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